**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 7/00 — A2

(11) International Publication Number: **WO 98/47913**

(43) International Publication Date: 29 October 1998 (29.10.98)

(21) International Application Number: PCT/US98/07533

(22) International Filing Date: 16 April 1998 (16.04.98)

(30) Priority Data:
08/844,448 18 April 1997 (18.04.97) US

(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application
US 08/844,448 (CIP)
Filed on 18 April 1997 (18.04.97)

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF MEDICINE AND DENTISTRY OF NEW JERSEY [US/US]; 30 Bergen Street, Newark, NJ 07107–3000 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WANG, Jihong [CN/US]; 101 Marvin Lane, Piscataway, NJ 08854 (US). STEIN, Stanley [US/US]; 6 Rowan Court, East Brunswick, NJ 08816 (US). LEIBOWITZ, Michael, J. [US/US]; 3 Baron Court, Manalapan, NJ 07726–2603 (US). RABSON, Arnold, B. [US/US]; 59 Marion Road East, Princeton, NJ 08540 (US).

(74) Agents: COHEN, Mark, S. et al.; Klauber & Jackson, 411 Hackensack Avenue, Hackensack, NJ 07601 (US).

(81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: INHIBITION OF HIV–1 REPLICATION BY A TAT RNA–BINDING DOMAIN PEPTIDE ANALOG

(57) Abstract

The peptidic compounds, R–Arg–Lys–Lys–Arg–Arg–Gln–Arg–Arg–Arg–X–(biotin)–$NH_2$ wherein R is the residue of a carboxylic acid and X is a cysteine or lysine residue, analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, contain the 9–amino acid sequence from the basic domain of the Tat protein responsible for specific interaction with TAR RNA, or an analog thereof. The cysteine or lysine residue provides an attachment site for biotin which acts as a cellular uptake enhancer. These peptides bind a fragment of TAR RNA (ΔTAR) avidly and specifically, as measured in an electrophoretic gel shift assay. Further, they inhibit *tat* gene–induced expression of a stably transfected CAT (chloramphenicol acety transferase) reporter gene linked to the HIV–1 LTR in a model cell assay, but do not inhibit phorbol ester–induced expression of CAT, thereby demonstrating a Tat–dependent mechanism of inhibition. Inhibition of HIV–1 replication after acute infection of MT2 cells was demonstrated by absence of HIV–induced syncytium formation and cytotoxicity, as well as by suppression of reverse transcriptase production. These results indicate that these peptides are capable of competing with the TAR RNA–binding domain of Tat protein and thus are useful as therapeutic agents in the treatment of AIDS.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **AL** | Albania | **ES** | Spain | **LS** | Lesotho | **SI** | Slovenia |
| **AM** | Armenia | **FI** | Finland | **LT** | Lithuania | **SK** | Slovakia |
| **AT** | Austria | **FR** | France | **LU** | Luxembourg | **SN** | Senegal |
| **AU** | Australia | **GA** | Gabon | **LV** | Latvia | **SZ** | Swaziland |
| **AZ** | Azerbaijan | **GB** | United Kingdom | **MC** | Monaco | **TD** | Chad |
| **BA** | Bosnia and Herzegovina | **GE** | Georgia | **MD** | Republic of Moldova | **TG** | Togo |
| **BB** | Barbados | **GH** | Ghana | **MG** | Madagascar | **TJ** | Tajikistan |
| **BE** | Belgium | **GN** | Guinea | **MK** | The former Yugoslav Republic of Macedonia | **TM** | Turkmenistan |
| **BF** | Burkina Faso | **GR** | Greece | | | **TR** | Turkey |
| **BG** | Bulgaria | **HU** | Hungary | **ML** | Mali | **TT** | Trinidad and Tobago |
| **BJ** | Benin | **IE** | Ireland | **MN** | Mongolia | **UA** | Ukraine |
| **BR** | Brazil | **IL** | Israel | **MR** | Mauritania | **UG** | Uganda |
| **BY** | Belarus | **IS** | Iceland | **MW** | Malawi | **US** | United States of America |
| **CA** | Canada | **IT** | Italy | **MX** | Mexico | **UZ** | Uzbekistan |
| **CF** | Central African Republic | **JP** | Japan | **NE** | Niger | **VN** | Viet Nam |
| **CG** | Congo | **KE** | Kenya | **NL** | Netherlands | **YU** | Yugoslavia |
| **CH** | Switzerland | **KG** | Kyrgyzstan | **NO** | Norway | **ZW** | Zimbabwe |
| **CI** | Côte d'Ivoire | **KP** | Democratic People's Republic of Korea | **NZ** | New Zealand | | |
| **CM** | Cameroon | | | **PL** | Poland | | |
| **CN** | China | **KR** | Republic of Korea | **PT** | Portugal | | |
| **CU** | Cuba | **KZ** | Kazakstan | **RO** | Romania | | |
| **CZ** | Czech Republic | **LC** | Saint Lucia | **RU** | Russian Federation | | |
| **DE** | Germany | **LI** | Liechtenstein | **SD** | Sudan | | |
| **DK** | Denmark | **LK** | Sri Lanka | **SE** | Sweden | | |
| **EE** | Estonia | **LR** | Liberia | **SG** | Singapore | | |

# INHIBITION OF HIV-1 REPLICATION BY A TAT RNA-BINDING DOMAIN PEPTIDE ANALOG

## BACKGROUND OF THE INVENTION

In view of the synergistic effect of inhibitors of HIV-1 reverse transcriptase and protease, it is reasonable to assume that a multi-drug/multi-target strategy will be required to achieve an effective therapy for AIDS. One potential target is the HIV Tat transactivator. Although functional Tat transactivation is considered to be critical for efficient viral transcription and replication (1-7), there has been relatively little progress in developing drugs that interfere with the transactivation process (8-10). Furthermore, a Tat antagonist may also be clinically useful for suppressing the suspected toxic effects of Tat protein (11-13). Indeed, the generation of anti-Tat antibodies in HIV-1 positive individuals may be protective with respect to disease progression (14).

The HIV Tat protein strongly activates HIV transcription through its interactions with TAR region RNA (reviewed in 1-3). The TAR domain consists of the first 57 nucleotides of all virally encoded RNAs (15,16). The predicted TAR RNA secondary structure is a double-stranded stem with a 3-base bulge and a 6-base loop. HIV-1 Tat is a small nuclear protein containing 86-102 amino acids encoded by multiply spliced mRNA. The 3 base bulge in TAR RNA and several other flanking nucleotides are essential for Tat-TAR interaction. Tat protein apparently acts to promote transcription by binding through its basic domain to the 3-base bulge of TAR (17-20). This is accompanied by recruitment of host cellular factors, including Tat and TAR binding proteins, to the TAR RNA stem and 6-base loop, as well as to the complex of template DNA, transcription factors and RNA polymerase (21-29). Initiation of proviral gene expression appears to occur by activation of an NF-κB and/or Sp1-dependent promoter (7,30), resulting in production of viral transcripts at a sufficient level to provide synthesis of Tat protein, which then interacts with TAR to allow enhanced production of elongated HIV transcripts.

One approach to development of agents specifically blocking Tat-mediated transactivation is to use, as a competitive inhibitor, a peptidic compound that mimics the basic TAR-binding domain of Tat protein. It has previously shown that a 10-residue Tat peptide with an appended 4-mer antisense oligonucleotide can specifically bind to TAR RNA, as shown by its ability to stimulate RNase H-mediated cleavage at the site of oligonucleotide annealing to the 6-base single-stranded loop (31). In that study, RNase H cleavage did not occur when a single base change was made in either the 3-base bulge or the 6-base loop region of TAR RNA, indicting that both peptide and oligonucleotide moieties of the conjugate contributed to the binding to TAR. To screen potential binding inhibitors, an assay (32) which is based on competitive inhibition of the gel shift of a 27-mer fragment of TAR RNA (△TAR) by a Tat binding domain peptide having an appended 5-kDa polyethylene glycol tail (Tat-PEG) has been developed.

## BRIEF DESCRIPTION OF THE DRAWING FIGURES

FIGURE 1A is a photograph of the gel shift assay. The mobility of 0.2 nM radiolabelled △TAR (control) is greatly decreased in the presence of 50 nM Tat-PEG. With increasing concentration of Tat10-biotin (nM), progressively less △TAR is bound to Tat-PEG and more to Tat10-biotin, which produces a very small gel shift.

FIGURE 1B is a graph showing the fraction of △TAR bound to Tat-PEG at each concentration, either in the absence or presence of tRNA.

FIGURE 2 is a graph showing the effect of Tat10-biotin addition at different times on pAR(Tat)-induced CAT protein expression. Tat10-biotin was added to HLCE-D36 cells at a concentration of 50 $\mu$M at either 18 hours or 42 hours and cells were harvested for CAT protein assay at 42 hours or 62 hours, respectively, after transfection with pAR(Tat).

FIGURE 3 is a graph showing the effect of Tat10-biotin addition at different concentrations on pAR(Tat)-induced CAT protein expression and on cell viability. Tat10-biotin was added to HLCE-D36 cells at 44 hours and cells were harvested for CAT protein assay (filled circles) at 66 hours after transfection with pAR(Tat). Cell viability (open circles) was determined by the MTT assay.

FIGURE 4 is a comparison of the assay of productive infection in MT2 cells. Cells were infected with HIV-1 and treated with 100 $\mu$M Tat10-biotin, as indicated. Cells were harvested at 96 hours after infection and assayed for reverse transcriptase content (FIGURE 4A) and viability according to MTT assay (FIGURE 4B).

FIGURE 5 shows syncytium formation by MT2 cell after HIV-1 infection. Cells were treated with HIV-1 and/or Tat10-biotin as indicated and observed at 72 hours after infection.

FIGURE 6 shows the results of a CAT model cell assay.

FIGURE 7 shows the results of the effects of the compounds on cell viability.

## SUMMARY OF THE INVENTION

The present invention concerns Tat-inhibitory polypeptide derivatives. More particularly, this invention relates to biotinylated polypeptides of the formula I: R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-$NH_2$ (SEQ ID NO:1), wherein R is the residue of a carboxylic acid, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, which exhibit advantageous properties, including binding to $\Delta$TAR, inhibition of LTR-dependent reporter gene expression in a model cell assay and, finally, inhibition of HIV-1 replication, as determined as assays of HIV-induced syncytium formation, cytotoxicity and reverse transcriptase production.

Further, this invention provides a peptide of the formula: R-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$,wherein R is the residue of a carboxylic acid, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof. In one embodiment, R is an acetyl group.

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention there is provided polypeptide of the formula I: R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-$NH_2$ (SEQ ID1), wherein R is the residue of a carboxylic acid, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, which are useful in the treatment of HIV-1 infection by virtue of their ability to block the interaction of Tat protein with TAR RNA, thereby interfering with the transactivation step in the replication cycle of HIV-1. The rationale for this approach is that such peptides compete with the full length Tat protein for binding to TAR RNA, thereby preventing the required interactions between other domains in Tat protein and the nascent transcription apparatus.

Indeed, cell culture experiments presented here using promoter elements of the HIV-1 LTR linked to the reporter CAT gene demonstrate that the peptides of the present invention containing the 9-amino acid basic domain of Tat protein block the transactivation process. The failure of a representative peptide of formula I, *i.e.*, Tat10-biotin (SEQ ID2), to block phorbol ester-induced transactivation indicates that this anti-transcription effect is due to inhibition of Tat protein activity rather than to non-specific effects on cellular transcription. The lack of toxicity further supports this hypothesis.

The utility of the peptides of formula I is further demonstrated by their ability to inhibit replication of HIV-1 in an acute infection assay.

A similar approach has been reported (40) using the cationic peptide, N-acetyl-$(D\text{Arg})_9$-$NH_2$. Antiviral activity of that peptide, however, was found to be via inhibition of viral entry, consistent with antiviral activity being observed when the cells were pretreated with peptide 24 hours prior to infection. In that study, the possibility of inhibition of transactivation by blocking Tat protein was suggested, but not proven, and no evidence of the peptide displaying specificity for TAR RNA was presented.

In the instant invention, there is convincing evidence from the HLCE-D36 cell assay that a representative peptide of formula I, Tat10-biotin, specifically blocks Tat protein-mediated expression of CAT protein. Although the mechanism whereby Tat protein induces protein expression from the HIV LTR is generally believed to be due to an effect on transcriptional elongation (2, 3, 7), it is possible that there are also post-transcriptional effects of Tat protein. Thus, whether Tat10-biotin competes with Tat protein only at the transcriptional elongation step remains to be determined.

In addition to demonstrating that a peptide antagonist of Tat protein can be utilized as an anti-HIV-1 agent, the instant invention also describes a series of assays that can be employed to aid in the development of an optimal analog for use as a therapeutic agent targeted to TAR RNA. The gel shift assay allows the evaluation of analogs of the Tat peptide for improved specificity and avidity of binding to TAR RNA. The use of radiolabelled and biotinylated derivatives provides a means for measuring, and thereby improving the parameters of cell uptake and peptide stability. The previously reported (40) nonapeptide containing the unnatural D-arginine residues might be exceptionally resistant to degradation by peptidases. However, in view of the observed antiviral activity of the D-arginine nonapeptide being due to blockage of viral entry rather than inhibition of Tat, demonstration of specificity of binding to TAR as opposed to non-specific binding to host cell RNA or to other cellular components is needed. By testing candidate inhibitory peptides for inhibition of transactivation in cells containing genetic elements of HIV-1, in addition to an acute infection paradigm, it can be confirmed that

any Tat peptide analog being studies operates via the mechanism of blocking interaction of Tat protein with TAR RNA.

The instant invention thus provides a method of treating a retroviral infection in a mammal in need of such treatment. More particularly, the peptides of the present invention can be utilized as Tat protein RNA-binding domain mimics to treat the HIV-1 infection, and the resultant AIDS. The mammal under treatment can be a human, monkey, cat or the like, with the treatment of humans being particularly preferred. A Tat antagonist should also be useful for ameliorating the pathogenic effects of Tat protein on host cells due to interactions with TAR-like elements on cellular transcripts (41). Recent studies (42) on peptide analogs of the core domain sequence of Tat protein, which is believed to interact with host cell factors rather than with virally encoded RNA, have lead to the same suggestion for a new class of therapeutic agents for AIDS based on inhibition of the transactivation step in the HIV-1 replication cycle.

The polypeptides of formula I and their analogs can be synthesized by conventional solution methods, or by solid phase synthetic techniques known in the art.

Throughout the specification and appended claims, the polypeptide of formula I, and its analogs and salts, encompass all stereo, optical and geometrical isomers thereof where such isomers exist, as well as the pharmaceutically acceptable salts and solvates thereof. Where appropriate, the polypeptide or its analogs can be utilized as its corresponding amide form. The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide.

The R group of the peptides of formula I can be any residue of an alkyl, alkenyl or aryl carboxylic acid, *i.e.*, acetic, propionic, butyric, valeric, allylic, benzoic and the like

being suitable. Particularly preferred for use in the present invention is the acetyl derivatives of the peptides of formula I.

The term "biologically and pharmaceutically acceptable salts" is intended to include any such salt derived from an inorganic or organic acid which is tolerated by the mammalian system. These salts include, but are not limited to, acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, hexanoate, succinate, fumarate, hydrochloride, hydrobromide, lactate, maleate, phosphate, sulfate, methanesulfonate, oxalate, propionate, tosylate, and mesylate. Examples of acids which can be used to form such salts include such inorganic acids as hydrochloric acid, sulfuric acid and phosphoric acid, and such organic acids such as oxalic acid, maleic acid, succinic acid and citric acid.

The nomenclature used to define the polypeptides is that specified by Schroder & Lubke, "The Peptides", Academic Press (1965), wherein in accordance with conventional representation the amino group at the N-terminal appears to the left and the carboxyl group at the C-terminal to the right. $NH_2$ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. NH2 also refers to the carboxyamide at the carboxy terminus of a polypeptide

Accordingly, polypeptide analogs displaying substantially equivalent activity to the polypeptide of formula I are likewise contemplated for use in the present invention. These modifications can be obtained through peptide synthesis utilizing the appropriate starting material.

Also, the term "active agent," "active ingredient" and "active medicament" are intended to include within their scope the polypeptide of formula I specifically recited herein as well as all substantially homologous analogs and allelic variations thereof.

In keeping with standard polypeptide nomenclature, *J. Biol. Chem.*, **243**:3552-59 (1969), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

| SYMBOL | | AMINO ACID |
|---|---|---|
| 1-Letter | 3-Letter | |
| Y | Tyr | tyrosine |
| G | Gly | glycine |
| F | Phe | phenylalanine |
| M | Met | methionine |
| A | Ala | alanine |
| S | Ser | serine |
| I | Ile | isoleucine |
| L | Leu | leucine |
| T | Thr | threonine |
| V | Val | valine |
| P | Pro | proline |
| K | Lys | lysine |
| H | His | histidine |
| Q | Gln | glutamine |
| E | Glu | glutamic acid |
| W | Trp | tryptophan |
| R | Arg | arginine |
| D | Asp | aspartic acid |
| N | Asn | asparagine |
| C | Cys | cysteine |

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the

beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues. The above Table is presented to correlate the three-letter and one-letter notations which may appear alternately herein.

An amino acid in the polypeptide of this invention can be changed in a non-conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting polypeptide. The present invention should be considered to include analogs whose sequences contain conservative changes which do not significantly alter the activity or binding characteristics of the resulting polypeptide.

The following is one example of various groupings of amino acids:

Amino acids with nonpolar R groups

Alanine
Valine
Leucine
Isoleucine
Proline
Phenylalanine
Tryptophan
Methionine

Amino acids with uncharged polar R groups

Glycine
Serine
Threonine
Cysteine
Tyrosine
Asparagine
Glutamine

Amino acids with charged polar R groups (negatively charged at PH 6.0)

Aspartic acid
Glutamic acid

Basic amino acids (positively charged at pH 6.0)

Lysine
Arginine
Histidine (at pH 6.0)

Another grouping may be those amino acids with aromatic groups:

Phenylalanine
Tryptophan
Tyrosine

Another grouping may be according to molecular weight (i.e., size of R groups):

Glycine 75

| | | |
|---|---|---|
| Alanine | | 89 |
| Serine | | 105 |
| Proline | | 115 |
| Valine | 117 | |
| Threonine | | 119 |
| Cysteine | | 121 |
| Leucine | | 131 |
| Isoleucine | | 131 |
| Asparagine | | 132 |
| Aspartic acid | | 133 |
| Glutamine | | 146 |
| Lysine | | 146 |
| Glutamic acid | 147 | |
| Methionine | | 149 |
| Histidine (at pH 6.0) | 155 | |
| Phenylalanine | 165 | |
| Arginine | | 174 |
| Tyrosine | | 181 |
| Tryptophan | | 204 |

Particularly preferred substitutions are:

- Gln for Arg or Lys; and
- His for Lys or Arg.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar

structure, which induces β-turns in the polypeptide's structure. Alternately, D-amino acids can be substituted for the L-amino acids at one or more positions.

Representative analogs of the polypeptide of formula I thus include:

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-$NH_2$ (SEQ ID NO:2)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-$NH_2$ (SEQ ID NO:3)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Cys-(biotin)-$NH_2$ (SEQ ID NO:4)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-$NH_2$ (SEQ ID NO:5)

N-acetyl-Gln-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-D-Lys-(biotin)-$NH_2$ (SEQ ID NO:6)

N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys(biotin)-$NH_2$ (SEQ ID NO:7)

In addition, this invention provides a peptide of the formula: R-(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$,wherein R is the residue of a carboxylic acid, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof. In one embodiment, R is an acetyl group.

This compound is composed of all D-amino acids for the purpose of stability against degradation; that is, to make it long-lasting *in vivo*. Since the side chains are all pointing in the wrong direction when L-amino acids are replaced by D-amino acids (inverso or enantio effect), the side chains can be reoriented to their correct positions by reversing the sequence (retro effect). Another way to look at it is that all the side chains in the retro-inverso peptide are in the correct spatial positions when binding to their target, which is TAR RNA of HIV-1, but the underlying peptide backbone is in the opposite direction. The retro-inverso approach works when only the side chains are important for interacting with the target and the peptide backbone is not important for interacting with the target, which appears to be true in this case.

Further, this invention provides, a pharmaceutical composition comprising a peptide of the formula R-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$, wherein R is the residue of a carboxylic acid, and analogs thereof, and the

biologically and pharmaceutically acceptable salts thereof, and a pharmaceutically acceptable carrier therefor.

Also, this invention provides a method of inhibiting HIV-1 replication which comprises administration a pharmaceutical composition comprising a peptide of the formula R-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$, wherein R is the residue of a carboxylic acid, and analogs thereof, in an amount so as to inhibit HIV-1 replication and a pharmaceutically acceptable carrier or diluent thereof.

As used herein, "pharmaceutical composition" means a therapeutically effective amounts of the peptides or compounds described herein together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers useful in SCF (stem cell factor) therapy. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance of SCF. The choice of compositions will depend on the physical and chemical properties of the protein having SCF activity. For example, a product derived from a membrane-bound form of SCF may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate

compositions coated with polymers (e.g., poloxamers or poloxamines) and SCF coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

Further, as used herein "pharmaceutically acceptable carrier" are well known to those skilled in the art and include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

The active peptide compound for use in the present invention can be, and is preferably, administered as a medicament, i.e., a pharmaceutical composition. As discussed earlier, the polypeptides and their analogs of the present invention, may be prepared in pharmaceutical compositions, with a suitable carrier and at a strength effective for administration by various means to a patient experiencing an adverse medical condition associated with HIV infection and/or AIDS for the treatment thereof. A variety of administrative techniques may be utilized, among them parenteral techniques such as subcutaneous, intravenous and intraperitoneal injections, catheterizations delivery across mucous membranes, and the like. Average quantities of the polypeptide or its

analogs may vary and in particular should be based upon the recommendations and prescription of a qualified physician.

The pharmaceutical compositions used in the methods of this invention for administration to animals and humans comprise the active compound in combination with a pharmaceutical carrier or excipient.

The medicament can be in the form of tablets (including lozenges and granules), dragees, capsules, pills, ampoules, intranasal sprays, or suppositories comprising the compound of the invention.

"Medicament" as used herein means physically discrete coherent portions suitable for medical administration. "Medicament in dosage unit form" as used herein means physically discrete coherent units suitable for medical administration, each containing a daily dose or a multiple (up to four times) or a sub-multiple (down to a fortieth) of a daily dose of the active compound of the invention in association with a carrier and/or enclosed within an envelope. Whether the medicament contains a daily dose, or, for example, a half, a third or a quarter of a daily dose will depend on whether the medicament is to be administered once, or, for example, twice three times or four times a day, respectively.

Advantageously, the compositions are formulated as dosage units, each unit being adapted to supply a fixed dose of active ingredients. Tablets, coated tablets, capsules, ampoules, intranasal sprays and suppositories are examples of preferred dosage forms according to the invention. It is only necessary that the active ingredient constitute an effective amount, i.e., such that a suitable effective dosage will be consistent with the dosage form employed in single or multiple unit doses. The exact individual dosages, as well as daily dosages, will, of course, be determined according to standard medical principles under the direction of a physician.

The active compound can also be administered as suspensions, solutions and emulsions of the active compound in aqueous or non-aqueous diluents, syrups, granulates or powders.

Diluents that can be used in pharmaceutical compositions (e.g., granulates) containing the active compound adapted to be formed into tablets, dragees, capsules and pills include the following: (a) fillers and extenders, e.g., starch, sugars, mannitol and silicic acid; (b) binding agents, e.g., carboxymethyl cellulose and other cellulose derivatives, alginates, gelatine and polyvinyl pyrrolidone; (c) moisturizing agents, e.g., glycerol; (d) disintegrating agents, e.g., agar-agar, calcium carbonate and sodium bicarbonate; (e) agents for retarding dissolution, e.g., paraffin; (f) resorption accelerators, e.g., quaternary ammonium compounds; (g) surface active agents, e.g., cetyl alcohol, glycerol monostearate; (h) adsorptive carriers, e.g., kaolin and bentonite; (i) lubricants, e.g., talc, calcium and magnesium stearate and solid polyethylene glycols.

The tablets, dragees, capsules and pills comprising the active compound can have the customary coatings, envelopes and protective matrices, which may contain opacifiers. They can be so constituted that they release the active ingredient only or preferably in a particular part of the intestinal tract, possibly over a period of time. The coatings, envelopes and protective matrices may be made, for example, from polymeric substances or waxes.

The active ingredient can also be made up in microencapsulated form together with one or several of the above-mentioned diluents.

The diluents to be used in pharmaceutical compositions adapted to be formed into suppositories can, for example, be the usual water-soluble diluents, such as polyethylene glycols and fats (e.g., cocoa oil and high esters, (e.g., $C_{14}$-alcohol with $C_{16}$-fatty acid]) or mixtures of these diluents.

The pharmaceutical compositions which are solutions and emulsions can, for example, contain the customary diluents (with, of course, the above-mentioned exclusion of solvents having a molecular weight below 200, except in the presence of a surface-active agent), such as solvents, dissolving agents and emulsifiers. Specific non-limiting examples of such diluents are water, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (for example, ground nut oil, glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitol or mixtures thereof.

For parenteral and intranasal administration, solutions and suspensions should be sterile, e.g., water or arachis oil contained in ampoules and, if appropriate, blood-isotonic.

The pharmaceutical compositions which are suspensions can contain the usual diluents, such as liquid diluents, e.g., water, ethyl alcohol, propylene glycol, surface active agents (e.g., ethoxylated isostearyl alcohols, polyoxyethylene sorbitols and sorbitan esters), microcrystalline cellulose, aluminum methahydroxide, bentonite, agar-agar and tragacanth, or mixtures thereof.

The pharmaceutical compositions can also contain coloring agents and preservatives, as well as perfumes and flavoring additions (e.g., peppermint oil and eucalyptus oil, and sweetening agents, (e.g., saccharin and aspartame).

The pharmaceutical compositions will generally contain from 0.5 to 90% of the active ingredient by weight of the total composition.

In addition to the active compound, the pharmaceutical compositions and medicaments can also contain other pharmaceutically active compounds.

Any diluent in the medicaments of the present invention may be any of those mentioned above in relation to the pharmaceutical compositions. Such medicaments may include solvents of molecular weight less than 200 as the sole diluent.

It is envisaged that this active compound will be administered perorally, intranasally, parenterally (for example, intramuscularly, intrathecally, intraperitoneally, subcutaneously, transdermally or intravenously), rectally or locally, preferably intranasally or parenterally, especially perlingually, or intravenously. Most preferably, the peptide of formula I, or its analog or salt, is administered by the intranasal or intravenous route.

In one embodiment the dosage rate, is in the range of 1.0 to 40 mg/kg of body weight. In another embodiment the dosage rate, is in the range of 1.0 to 30 mg/kg of body weight. In another embodiment the dosage rate, is in the range of 1.0 to 20 mg/kg of body weight. In another embodiment the dosage rate, is in the range of 2.0 to 15 mg/kg of body weight. The dosage rate, is preferably in the range of 0.01 to 20 mg/kg of body weight, and most preferably in the range of 0.5 to 5 mg/kg of body weight, and will be a function of the nature and body weight of the subject to be treated, the individual reaction of this subject to the treatment, type of formulation in which the active ingredient is administered, the mode in which the administration is carried out and the point in the progress of the disease or interval at which it is to be administered. Thus, it may in some case suffice to use less than a minimum dosage rate, while other cases an upper limit must be exceeded to achieve the desired results. Where larger amounts are administered, it may be advisable to divide these into several individual administrations over the course of the day. In this regard, the intranasal administration may utilize metered dose devices known in the art. In a preferred embodiment, the route of administration is oral. As discussed below, the biotin moiety enhances intestinal uptake of orally administered antiviral compound. Lastly, this invention provides a useful model for studying cellular and molecular regulation of biotin uptake.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

## EXAMPLES

### EXAMPLE 1

Peptide Synthesis:

Tat10-(K)biotin (N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys(biotin)-$NH_2$) (SEQ ID NO:3) and Tat10-biotin, N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys(biotin)-$NH_2$ (SEQ ID NO:2), were synthesized manually on PAL resin by Fmoc chemistry with reagents from PerSeptive Biosystems (Framingham, MA). Fmoc-Lys(biotin) was obtained from Bachem (Torrance, CA). Biotin was appended to the cysteine thiol group through a thioether bond using the reagent, iodoacetyl-LC-biotin (Pierce, Rockford, IL), as follows. Following assembly of the peptide and while still attached to the solid support, the trityl protecting group was removed from the cysteine sulfur atom by treatment with 1% trifluoroacetic acid (TFA) in dichloromethane for 2 hours at room temperature. The solid support was washed with dimethylformamide (DMF) and iodoacetyl-LC-biotin (4- mg/ml) in DMF was added at a 5-fold molar excess. Conjugation with biotinylation reagent proceeded overnight with vigorous shaking. Peptides were cleaved from the support and deprotected by 4 hours treatment with a mixture of 90% TFA, 5% thioanisole, 2% anisole and 3% ethanedithiol. Peptides were purified by reverse-phase HPLC and characterized by mass spectrometry for the molecular ion. Peptide concentration was determined by amino acid analysis.

### EXAMPLE 2

Binding Assay:

As previously reported by Wang *et al.*, *Anal. Biochem.* 1995; 232: 238-242 (32), $\Delta$TAR (+18 to +44 with respect to the transcription start site) was transcribed *in vitro*, gel purified and radiolabelled. The binding reaction mixtures (10 $\mu$l) contained 0.2 nM

5'-[$^{32}$P] ΔTAR, 50 nM Tat-PEG, varying concentrations of Tat10-biotin, 50 mM Tris HCl (pH 7.5), 70 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% NP40 and 10% glycol, in the presence or absence of 50 nM yeast tRNA as a competitor to evaluate non-specific binding. The reaction mixtures were incubated at 30° C for 15 minutes prior to electrophoresis in nondenaturing 4% polyacrylamide gels (75:1 w/w acrylamide: bisacrylamide). The gels were electrophoresed at 150 V for 40 minutes at 4° C in TBE buffer (49 mM Tris, 49 mM borate, 5 mM EDTA, pH 8.3). The gels were dried and the percentage of ΔTAR RNA probe shifted by Tat-PEG was quantitated by phosphorimager analysis (GS250, BioRad).

*Cell Uptake and Stability Analysis:*

Prior to the final acetylation step in peptide synthesis, a portion of the solid support, equivalent to about 3 mg (2 μmol) of peptide, was removed and reacted with 1 mCi (0.15 μmol) of tritiated acetic anhydride in the presence of peptide coupling activation reagents. Acetylation of the peptide was completed by chasing with an excess (50 μmol) of non-radioactive acetic anhydride. After washing the resin, the cysteine residue was biotinylated as described above. After cleavage from the solid support, the radiolabelled peptide was precipitated with ether and then purified by chromatography on Sephadex G-10. Non-biotinylated, radiolabelled peptide was also prepared in a similar manner.

For stability assays, radiolabelled Tat10-biotin was further purified by binding and extracting from avidin-coated beads. Essentially 100% of the radiolabel bound to avidin-coated beads in buffer or in culture medium containing 8% fetal calf serum and pretreated with the protease inhibitor, phenylmethylsulfonyl fluoride. For Tat10-biotin treated with trypsin, essentially none of the radiolabel bound to the avidin-coated beads. Loss of radiolabel binding to avidin-coated beads in a sample incubated with serum was, therefore, scored as peptide degradation.

For uptake studies, cells (5 X $10^6$) were incubated with either radiolabelled biotinylated or non-biotinylated peptide (5 nmol each). Nuclear, cytoplasmic and membrane fractions were prepared according to the procedure of Dyer and Herzog, *BioTechniques* 1995; 19:192-194 (33), with minor modifications.

*Model LTR-CAT Assays*:

Jurkat-derived HLCE-D36 cells are stably transfected with a recombinant plasmid containing the HIV-1 long terminal repeat (LTR) linked to the chloramphenicol acetyl transferase (CAT) gene on the pRep10 EBV episomal vector (Invitrogen). Cells were grown in RPMI 1640 medium supplemented with HEPES (10 mM), glutamine (4 MM), fetal bovine serum (10%), penicillin (50 U/ml), streptomycin (50 $\mu$g/ml) and hygromycin B (Boehringer-Mannheim, 0.3 mg/ml). For expression of Tat protein, cells were transfected with pAR(tat), which contains the HIV-1 LTR and viral sequences mapping between 5.7 and 6.3 kb, encoding the 72-amino acid first exon of Tat, Gendelman *et al.*, *Proc. Natl. Acad. Sci. USA* 1986; 83:9759-9763 (34). In this procedure, 2 x $10^7$ cells were suspended in 1 ml of serum-free RPMI medium containing 10 $\mu$l of DEAE dextran (Sigma Chemical Co.) solution (10 mg/ml in water), and then incubated with 10 $\mu$g of pAR(Tat) for 20 minutes at 37°C in 5% $CO_2$ Dorset, *et al.*, *J. Virol.* 1983; 48:218-228 (35). Cells were washed in serum-free RPMI medium and then resuspended in RPMI medium containing hygromycin B (0.3 mg/ml) at a density of 3.5 x $10^5$ cells/ml. Equal aliquots of the cells (0.6 ml/well) were then added to a 24-well microtiter plate and maintained at 37°C in 5% $CO_2$.

Tat10-biotin was added to the microtiter plate wells (in triplicate) at designated times after transfection at a final concentration of 50 $\mu$M, unless otherwise indicated. At each assay time point, an aliquot (0.1 ml) was removed from each control or peptide-treated well for determination of cell viability by MTT assay (Sigma Chemical Co.). The remainder of the cells from each well were collected and washed with phosphate-

buffered saline by centrifugation. Cells were lysed and CAT protein was measured using an ELISA kit (Boehringer-Mannheim).

*Anti-HIV-1 Assays*:

MT2 cells were seeded in 96-well plates at 20,000 cells per well and infected with HIV (LAI laboratory strain) at a multiplicity of infection of 0.01. The various experimental groups included: uninfected cells (mock), cells infected with HIV-1, cells infected with HIV-1 and treated twice with Tat10-biotin or the control peptide (100 $\mu$M) at t=0 and 48 hours and uninfected cells treated with Tat10-biotin (100 $\mu$M) at t=0 and 48 hours as for infected cells. Individual wells were analyzed (our n=5) at 96 hours for cell viability by the MTT assay of Tada *et al.*, *J. Immunol. Methods* 1986; 93:157-165 (37) and for reverse transcriptase by incorporation of label from [$^{32}$P]-TTP into TCA-precipitable material in the presence of an oligoA template, see, Willey *et al.*, *J. Virol.* 1988; 62:139-147 (38). Syncytium formation was observed by light microscopy 48-72 hours post-infection.

## RESULTS

*Binding Assay*

A radiolabelled 27-mer RNA, representing the Tat binding site of TAR RNA (△TAR), migrates as predicted by its molecular weight on a nondenaturing electrophoresis gel, as detected by phosphorimager analysis (FIGURE 1, control lane on gels). Migration of △TAR can be shifted to lower mobility in the presence of Tat protein (19) or, more reproducibly, by a 10-mer Tat peptide having a 5-kDa polyethylene glycol tail (Tat-PEG). In the presence of 50 nM Tat-PEG and with increasing amounts of the competitor peptide, Tat10-biotin, there is a decrease in the proportion of shifted △TAR (FIGURE 1). A similar effect is seen when the gel shift assay is done in the presence of a large excess (750-fold by weight) of tRNA relative to △TAR to determine the effect of nonspecific binding of peptides to unrelated RNA. FIGURE 1B presents the data from these experiments graphically, where binding inhibition in the presence or

absence of competitor tRNA is shown. The concentration of Tat10-biotin producing 50% inhibition of the gel shifted band was found to be 150 nM, representing 3-fold weaker binding ($K_d$ of 7.5 nM) than Tat-PEG ($K_d$ previously determined to be 2.5 nM). However, in the presence of tRNA, where the apparent $K_d$ for Tat-PEG is 27 nM, Tat10-biotin produced 50% inhibition of the gel shifted band at 45 nM, equivalent to a apparent $K_d$ of 24 nM. Thus, the presence of the biotin moiety results in weaker binding to $\Delta$TAR, but this effect is more than compensated for by a decrease in nonspecific binding to extraneous RNA.

*Uptake and Stability Analysis*

Although the biotin group has a favorable effect on TAR RNA binding in the presence of extraneous RNA, the original reason it was appended to the Tat peptide was to enhance cellular uptake, as described by Chen *et al*, *Anal. Biochem.* 1995; 227:168-175 (39). Radiolabelled Tat10-biotin (SEQ ID NO:2 and Tat10 N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-$NH_2$ (SEQ ID NO:8) were each prepared using tritiated acetic anhydride for the N-acetylation reaction. According to an uptake time course in Jurkat cells in serum-containing medium, about 0.1% and 3% of the label added to the culture was found in cells for the non-biotinylated and biotinylated peptides, respectively, after 2 hours. Nuclear localization was similar for the biotinylated and non-biotinylated peptides, being about 0.1% of the radioactivity added to the culture. The extent to which cytoplasmic localization represents lysosomal entrapment remains to be determined. No significant radiolabel was associated with the membrane fraction. The half-life of Tat10-biotin was about 80 minutes in culture medium containing 8% heat-inactivated serum.

*Model LTR-CAT Assays*

Tat10-biotin (SEQ ID NO:2) was tested for inhibition of CAT expression from the HIV LTR on an EBV episomal plasmid stably transfected into Jurkat T cells (HLCE-D36).

Tat10-biotin (50 $\mu$M) was added to the LTR-CAT containing cells at 18 hours after transfection with the Tat-expressing plasmid, pAR(tat), and the level of CAT protein was determined at 42 hours. A substantial decrease (58%) in CAT protein was observed for peptide-treated versus control cells (FIGURE 2). A similar result (54% decrease) was obtained for addition of Tat10-biotin at 42 hours and measurement of CAT at 62 hours. The lability of Tat10-biotin is evident from the recovery of CAT expression for peptide added at 18 hours and CAT protein assayed at 62 hours (FIGURE 2). Similar results were obtained in several separate experiments, including addition of Tat10-biotin at time points of 2 or 4 hours after transfection with pAR(Tat).

A dose-response experiment for Tat10-biotin (SEQ ID NO:2) indicated 50% of maximal inhibition of CAT protein level at a concentration of about 15 $\mu$M (FIGURE 3) when the peptide was added at 44 hours and the cells were harvested at 66 hours after transfection. Since the intracellular turnover rate of CAT protein is not known, the actual amount of inhibition of CAT protein synthesis cannot be calculated from this experiment. Thus, the 65% decrease in CAT protein in the presence of 150 $\mu$M Tat10-biotin (FIGURE 3) may actually represent 100% inhibition of *de novo* CAT protein synthesis. Although a side-by-side comparison of Tat10-biotin (SEQ ID NO:2) and Tat10 (SEQ ID NO:8) (i.e., non-biotinylated) was not done, the results from several separate assays of each indicated that a 2-3 fold higher concentration of Tat10 (SEQ ID NO:8) was required to achieve the same inhibition of CAT protein.

These results demonstrate that addition of Tat10-biotin can inhibit pAR(Tat)-induced CAT expression, even though a substantial intracellular concentration of Tat protein is already present. However, when phorbol myristic acid, rather than transfection with pAR(Tat), was used to induce CAT expression from the LTR promoter, no inhibitory effect was observed with Tat10-biotin (Table 1). This implies that the inhibitory effect of Tat10-biotin ensues from competition with Tat protein. Various control peptides found to have no significant inhibitory activity following pAR(Tat) transfection

included $(Lys)_3Arg$-$(Lys)_3$-$NH_2$, $(Lys)_3Arg$-$(Lys)_2$-Lys(ε-acridine)-$NH_2$ and N-acetyl-$(Arg)_5$-$NH_2$.

Table 1. Effect of Tat10-biotin on CAT protein expression induced by phorbol myristic acid (PMA)

| PMA Conc. (nM) | Time of Tat10-Biotin Addition | CAT Protein pg |
|---|---|---|
| Exp. 1 | | |
| 0 | no peptide | 1.0(.2) |
| 32 | no peptide | 36(1) |
| 32 | 0 | 46(3) |
| 32 | 2 | 38(2) |
| 32 | 4 | 38(1) |
| Exp. 2 | | |
| 0 | no peptide | 10(1) |
| 15 | no peptide | 148(9) |
| 15 | 0 | 146(4) |
| 15 | 3 | 146(9) |
| Exp. 3 | | |
| 0 | no peptide | 9(1) |
| 2.5 | 0 | 55(1) |
| 2.5 | 4 | 50(2) |

PMA was added to cells at t=0. Tat10-botin was added at indicated times at 50 μM. Cells were harvested at 24 hours and assayed for CAT protein. Each value is the average of 3 separate cell cultures, and the average deviations are given in parentheses.

*Anti-HIV-1 Assays*

Tat10-biotin (100 $\mu$M) was tested for possible inhibitory effects on productive HIV-1 infection of MT2 cells. Strong suppression of HIV-1 reverse transcriptase production to background levels was apparent at day 4 following infection (FIGURE 4A) in cells treated twice with Tat10-biotin at t=0 and 48 hours. Treatment of cells with a similarly biotinylated control peptide, N-acetyl-Arg-Arg-Arg-Cys(biotin)-$NH_2$, which has poor affinity for TAR RNA according to the gel shift assay, did not inhibit HIV production as compared to untreated cells (FIGURE 4A). HIV-1-induced cytotoxicity was also strongly inhibited by Tat10-biotin treatment, as measured by MTT assay (FIGURE 4B). Again, the control peptide did not block HIV-induced cytotoxicity (FIGURE 4B). The MTt assay further indicated that Tat10-biotin does not inhibit MT2 cell growth, suggesting that this peptide does not exhibit toxicity. Similar results were obtained for Tat10-biotin in a single addition at t=0 or in a triple addition at t=0, 48 and 72 hours.

Tat10-biotin treatment also inhibited HIV-induced syncytium formation. Pronounced syncytium formation was readily observable in infected cells not treated with Tat10-biotin (FIGURE 5), but no syncytia were observed in HIV-infected cells treated with Tat10-biotin or in the control uninfected MT2 cells (FIGURE 5). The inhibitory effects of Tat10-biotin on HIV infection in MT2 cells have been demonstrated in multiple independent experiments.

While the invention has been described and illustrated herein by references to various specific material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art.

EXAMPLE 3

To obtain an improved compound, the retro-inverso (also called the retro-enantio) analog, was prepared and tested the following:

Compound I.

N-acetyl-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$

This compound is composed of all D-amino acids for the purpose of stability against degradation; that is, to make it long-lasting *in vivo*. Since the side chains are all pointing in the wrong direction when L-amino acids are replaced by D-amino acids (inverso or enantio effect), the side chains can be reoriented to their correct positions by reversing the sequence (retro effect). Another way to look at it is that all the side chains in the retro-inverso peptide are in the correct spatial positions when binding to their target, which is TAR RNA of HIV-1, but the underlying peptide backbone is in the opposite direction. The retro-inverso approach works because only the side chains are important for interacting with the target and the peptide backbone is not important for interacting with the target.

Peptide Synthesis:

The retro-inverso peptide was synthesized manually on PAL resin using the customary conditions of Fmoc chemistry. The peptide synthesis reagents were from PerSeptive Biosystems (Framingham, MA). Fmoc-dLys(Mtt)-OH, obtained from Bachem Bioscience (King of Prussia, PA), was used only at the position in the peptide to be biotinylated. Following assembly of the peptide and while still on the resin, the Mtt protecting group was selectively removed by mild acid treatment. The resulting deprotected amino group was then reacted with an excess of biotin in the presence of peptide coupling reagents. The resin was washed and the peptide was cleaved from the resin and deprotected as usual. The retro-inverso peptide was purified by reverse-phase 11PLC. Structure of the retro-inverso peptide was confirmed by MALDI-TOF mass spectrometry for the molecular ion.

The retro-inverso peptide was synthesized and tested in the gel shift binding assay, Table II, and the CAT cells culture assay, both procedures described Choudhury et al. The retro-inverso is herein referred to as compund I.

Table II

GEL SHIFT BINDING ASSAY

| Compound | Krel | K'rel |
|---|---|---|
| I | 0.4 | 0.9 |
| II | 3 | 0.9 |
| III | 4 | 2 |
| IV | 0.5 | 6 |

Compound II is the compound studied in Choudhury et al., Tat10(biotin) which is as follows: N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg--Cys(biotin). Compound III is as follows: N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg--Lys(ϵ-biotin), from which the retro-inverso sequence is derived. Compound IV is from Sumner-Smith et al., which is as follows: N-acetyl-DArg-DArg-DArg-DArg-DArg-DArg-DArg-DArg-DArg-$NH_2$.

Compound I has the best combination of binding avidity (Krel) and specificity in the presence of excess unrelated RNA (K'rel), where the lower the number, the better the binding. The data for the CAT model cell assay is depicted in Figure 6.

In this experiment, the Tat inhibitory peptide was added to the cells at a single, relatively large dose of 50 $\mu$M at t = 4 hours, and the cells were harvested and assayed for CAT protein at the indicated times. Tat9K(biotin) (III) gave good inhibition of CAT expression in comparison with the control of no peptide added and the control of an ineffective peptide added (R3). Compounds I and IV both also inhibited CAT expression, but in a long-lasting manner, which is a desirable property.

An undesirable property is a toxic effect on cell viability, as shown in Figure 7. This experiment was done on a set of parallel samples as the previous study; the only difference was that the cells were tested for viability (MTT method mentioned in Choudhury et al.) instead of for CAT protein. The control peptide (R3) and III showed no appreciable effect on cell viability. Peptides made of D-amino acids did have a deleterious effect on cell viability, with the Sumner-Smith compound IV being more deleterious than compound I.

Based on the foregoing experiments, the biotin moiety has substantial and unexpected desirable properties. First, it enhances uptake of the compound into cells. In one study, the cell uptake in serum-containing culture medium was only 0.1% of added radiolabeled peptide without biotin, but 3% with the biotinylated peptide - quite a difference. Furthermore, the biotin moiety increases the specificity of binding of the Tat peptide to RNA. Even though the biotin moiety does somewhat decrease the binding avidity to its target, TAR RNA, its negative effect on nonspecific binding to extraneous RNA is even more pronounced. The net result is that more biotinylated Tat peptide vs non-biotinylated Tat peptide binds to a given amount of TAR RNA when there is an excess of unrelated RNA present, as shown in our gel shift assay using tRNA as an example of a nonspecific RNA. Indeed, the assay probably underestimates the beneficial effect on specific binding in the presence of extraneous RNA, since the typical cell contains a variety of different RNAs and in higher relative amounts than does our in vitro assay system.

Another point is that the placement of the biotin moiety in the peptide structure is important (Table III). If the biotin is placed at the other end of the peptide, the result is a substantial loss in binding affinity to TAR RNA (7-fold from a Krel of 3 for #3 to a Krel of 20 for #5 where the higher the value of Krel the weaker the binding to TAR RNA. Furthermore, the way the biotin moiety is linked to the Tat peptide is important. Using a disulfide linkage (#4) instead of a thioether linkage (#3) diminishes affinity for TAR RNA by about 5-fold. Also, you cannot just use any uptake enhancer. Cholic

acid moieties have been proposed as cell uptake enhancers, but in our case they were found to severely diminish target binding (#6 and #7). In Table III, the K'rel represents affinity to TAR RNA in the presence of a 750-fold (by weight) excess of nonspecific tRNA. Both Krel and K'rel must be as low as possible. That is why #1, which has a K'rel of 15, is not useful while #3, which is the same as SEQ ID2, is useful.

In the model cell assay of CAT expression, the biotinylated Tat peptide was found to be 3-times more potent than the non-biotinylated peptide. The difference in potency might be even more pronounced *in vivo* where all the beneficial properties of the appended biotin moiety can come into play. Most telling is that the best compound of Sumner-Smith et al., termed 4C or ALX40-4C, just does not get into cells but blocks entry of the virus (HIV-1) by binding to a certain receptor on the outside of the cell; unfortunately, it does not block entry of all viral variants making it potentially useless as a drug for AIDS.

Table III. Tat Peptides with uptake enhancers

| Compound | Peptides | Krel | Krel' | Abbreviations |
|---|---|---|---|---|
| | Tat10-PEG | 1 | 1 | |
| I. | K(ϵ-acridine)KKRKKK | 0.65 | 15 | Jp2 |
| II. | Biotin-RKKRRQRRRC-$NH_2$ | 20 | - | Biotin-Tat10 |
| III. | Ac-RKKRRQRRRC(LC-Biotin)-$NH_2$ | 3 | 0.9 | Tat10-biotin |
| IV. | Ac-RKKRRQRRRC(LC-Biotin)-$NH_2$ | 16 | 6 | Tat10-S-S-biotin |
| V. | Biotin-LC-RKKRRQRRRC-$NH_2$ | 20 | - | Biotin-LC-Tat10 |

| | | | | |
|---|---|---|---|---|
| VI. | Ac-RKKRRQRRRK(cholyl)-$NH_2$ | 50 | 50 | Tat9K(Ch) |
| VII. | Cholyl-RKKRRQRRRK(Biotin)-$NH_2$ | $>100$ | - | CH-Tat9K(biotin) |

In addition to proof of principle that a peptide antagonist of Tat protein can function as an anti-HIV-1 agent as disclosed herein a series of assays that can be used to aid in the development of an optimal analog for use as a therapeutic agent targeted to TAR RNA. The gel shift assay permits evaluation of analogs of the Tat peptide for improved specificity and avidity of binding to TAR RNA. The use of radiolabeled and biotinylated derivatives provides a means for measuring, and thereby improving, the parameters of cell uptake and peptide stability. In conclusion, the studies with Tat 10-biotin indicate the potential usefulness of Tat protein RNA-binding domain mimics as therapeutic agents for AIDS. A Tat antagonist is also useful for ameliorating the pathogenic effects of tat protein on host cells due to interactions with TAR-like elements on cellular transcript.

The following is a list of documents related to the above disclosure and particularly to the experimental procedures and discussions. The documents should be considered as incorporated by reference in their entirety.

1. Cullen, B.R. Mechanism of action of regulatory proteins encoded by complex retroviruses. *Microbiol. Rev.* 1992; 56:375-394

2. Jones, K.A. and Peterlin, B.M. Control of RNA initiation and elongation at the HIV-1 promoter, *Ann. Rev. Biochem.* 1994; 63:717-743

3. Gaynor, R.B. Regulation of HIV-1 gene expression by the transactivator Protein Tat. *Curr. Top. Microbiol. Immunol.* 1995; 193:51-77

4. Fisher, A. G.; Feinberg, M.B.; Josephs, S.F.; Harper, M.E.; Marselle, L.M.; Reyes, G.; Gonda, M.A.; Aldovini, A.; Debouk, C.; Gallo, R.C.; and Wong-Staal, F. The *trans*-activator of HTLV-III is essential for virus replication. *Nature* 1986; 320:367-371

5. Dayton, A.I.; Sodroski, J.G.; Rosen, C.A.; Goh, W.C.; and Haseltine, W.A. The *trans*-activator gene of the human T-cell lymphotropic virus type III is required for replication. *Cell* 1986; 44:941-947

6. Harrich, D.; Hsu, C.; Race, E.; and Gaynor, R.B. Differential growth kinetics are exhibited by human immunodeficiency virus type 1 TAR mutants. *J. Virol.* 1994; 68:5899-5910

7. Feinberg, M.B.; Baltimore, D.; and Frankel, A.D. The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. *Proc. Natl. Acad. Sci. USA* 1991; 88:4045-4049

8. Cupelli, L.A. and Hsu, M-C. The human immunodeficiency virus type 1 Tat antagonist, Ro 5-3335, predominantly inhibits transcription initiation from the viral promoter. *J. Virol.* 1995; 69:2640-2643

9. Farese, A.; Peytou, V.; Condom, R.; Sinet, M.; Patino, N.; Kirn, A.; Moog, C.; Aubertin, A.M.; and Guedj, R. Synthesis of analogues of the benzodiazepine Ro 5-3335, antagonist of Tat HIV-1. Biological evaluation by Luciferase transactivation and anti-viral assay. *Eur. J. Med. Chem.* 1996; 31:497-505

10. Lapidot, A.; Ben-Asher, E.; and Eisenstein, M.. Tetrahydropyrimidine derivatives inhibit binding of a Tat-like arginine-containing peptide to HIV TAR RNA. *FEBS Lett.* 1995; 367:33-38

11. Mc Millan, N.A.J.; Chun, R.F.; Siderovski, D.P.; Galabrus, J.; Toone, M.; Samuel, C.E.; Mak, T.W.; Hovanessian, A.G.; Jeang, T.K.; and Williams, B.R.G. HIV-1 Tat directly interacts with the interferon-induced double-stranded RNA-dependent kinase, PKR. *Virology* 1995; 213:413-424

12. Brady, H.J.M.; Abraham, D.J.; Pennington, D.J.; Miles, C.G.; Jenkins, S.; and Dzierzak, E.A. Altered cytokine expression in T lymphocytes from human immunodeficiency virus Tat transgenic mice. *J. Virol.* 1995; 69:7622-7629

13. Li, C.J.; Friedman, D.J.; Wang, C.; Metelev, V.; and Pardee, A.B. Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein. *Science* 1995; 268:429-431

14. Re, M.C.; Furlini, G.; Vignoli, M.; Ramazzotti, E.; Roderigo, G.; De Rosa, V.; Zauli, G.; Lolli, S.; Capitani, S.; and La Placa, M. Effect of antibody to HIV-1 Tat protein on viral replication in vitro and progression of HIV-1 disease *in vivo*. *J. Acq. Imm. Def. Syn. and Human Retrovirol.* 1995; 10:408-416

15. Hauber, J. and Cullen, B.R. Mutational analysis of the transactivation-responsive region of the human immunodeficiency virus type I long terminal repeat. *J. Virol.* 1988; 62:673-679

16. Jacobovits, D.; Smith, H.; Jakobovits, E.B.; and Capon, D.J. A discrete element 3' of human immunodeficiency virus 1 (HIV-1) and HIV-2 mRNA initiation sites mediates transcriptional activation by an HIV *trans* activator. *Mol Cell Biol.* 1988; 8:2555-2561

17. Dingwall, C.; Ernberg, I.; Gait, M.J.; Green, S.M., Heaphy, S.; Karn, J.; Lowe, A.D.; Singh, M.; Skinner, M.A.; and Valerio, R. Human immunodeficiency virus 1 tat protein binds trans-activation-responsive region (TAR) RNA *in vitro*. *Proc. Natl. Acad. Sci. U.S.A.* 1989; 86:6925-6929

18. Cordingly, M.G.; Lafemina, R.L.; Callahan, P.L.; Condra, J.H.; Sardana, V.V.; Graham, D.J.; Nguyen, T.M.; LeGrow, K.; Gotlib, L.; Schlabach, A.J.; and Colonno, R.J. Sequence specific interaction of Tat protein and at peptides with the transactivation-response sequence element of human immunodeficiency virus type 1 *in vitro*. *Proc. Natl. Acad. Sci. USA*. 1990; 87:8985-8989.

19. Roy, S.; Delling, U.; Chen, C.H; Rosen, C.A.; and Sonenberg, N. A bulge structure in HIV-1 TAR RNA is required for Tat binding and Tat-mediated tran-activation. *Genes Dev.* 1990; 4:365-1373

20. Weeks, K.M.; Ampe, C; Schultz, S.C.; Steitz, T.A.; and Crothers, D.M. Fragments of the HIV-1 Tat protein specifically bind TAR RNA *Science* 1990; 249:1281-1285

21. Marciniak, R.A.; Garcia-Blanco, M.A.; and Sharp, P.A. Identification and characterization of a HeLa nuclear protein that specifically binds to the trans-activation-

response (TAR) element of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* 1990; 87:3624-3628

22. Sheline, C.T.; Milocco, L.H.; and Jones, K.A. Two distinct nuclear transcription factors recognize loop and bulge residues of the HIV-1 TAR RNA hairpin. *Genes Dev.* 1991; 5:2580-2520

23. Wu, F.; Garcia, J.; Sigman, D.; and Gaynor, R. Tat regulates binding of the human immunodeficiency virus trans-activating region RNA loop-binding protein TRP-185. *Genes Dev.* 1991; 5:2128-2140

24. Gatignol, A; Buckler-White, A.; Berkhout, B.; and Jeang, K.T. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. *Science* 1991; 251:1597-1600

25. Shibuya, H.; Irie, K.; Ninomiya-Tsuji, J.; Goebl, M.; Taniguchi, T.; and Matsumoto, K. New human gene encoding a positive modulator of HIV tat-mediated transactivation. *Nature* 1992; 358:700-702

26. Desai, K.; Loewenstein, P.M.; and Green, M. Isolation of a cellular protein that binds to the human immunodeficiency virus tat protein and can potentiate transactivation of the viral promoter. *Proc. Natl. Acad. Sci USA* 1991; 88:8875-8879

27. Ohana, B.; Moore, P.A.; Ruben, S.M.; Southgate, D.; Green, M.E.; and Rosen, C.A. The type 1 human immunodeficiency virus Tat binding protein is a transcriptional activator belonging to an additional family of evolutionariiy conserved genes. *Proc. Natl. Acad. Sci USA* 1993; 90:138-142

28. Herrmann, C.R. and Rice, A.P. Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxy-terminal

domain of the large subunit of RNA polymerase II: candidate for Tat cofactor. *J. Virol.* 1995; 69:1612-1620.

29. Mavankal, G.; Ou, SHI; Oliver, H; Sigman, D.; and Gaynor, R.B. Human immunodeficiency virus type 1 and 2 Tat proteins specifically interact with RNA polymerase II. *Proc. Natl. Acad. Sci. USA* 1996; 93:2089-2094

30. Antoni, B.A.; Stein, S.B.; and Rabson, A.B. Regulation of human immunodeficiency virus infection: implications for pathogenesis. In: K. Mamarosch; F.A. Murphy; and A.J. Shatkin, eds. *Adv. Virus Res* Academic Press. 1994; 43:53-145

31. Tung, C-H; Wang, J.; Leibowitz, M.J.; and Stein, S. Dual specificity interactions of HIV-1 TAR RNA with Tat peptide-oligonucleotide conjugates. *Bioconj. Chem.* 1995; 6:292-295

32. Wang, J.; Huang, S-Y; Choudhury, I.; Leibowitz, M.J.; and Stein, S. Use of a polyethylene glycol-peptide conjugate in a competition gel shift assay for screening potential antagonists of HIV-1 Tat protein binding to TAR RNA. *Anal. Biochem.* 1995; 232:238-242

33. Dyer, R.B. and Herzog, N.K. Isolation of intact nuclei for nuclear extract preparation from a fragile B-lymphocyte cell line. *BioTechniques* 1995; 19:192-194

34. Gendelman, H.E.; Phelps, W.; Feigenbaum, L.; Ostrove J.M.; Adachi, A.; Howley, P.M.; Khoury, G.; Ginsberg, H.S.; and Martin, M.A. Trans-activation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. *Proc. Natl. Acad. Sci. USA* 1986; 83:9759-9763

35. Dorset, D.L.; Keshet, I.; and Winocour, E. Quantitation of a simian virus 40 nonhomologous recombination pathway. *J. Virol.* 1983; 48:218-228

36.Lehrach, H.; Diamond, D.; Wozney, J.M.; and
Boedtker, H. RNA molecular weight determinations by get electrophoresis under denaturing conditions, a critical re-examination. *Biochemistry* 1977; 16:4773

37. Tada, H.; Shiho, O.; Kuroshima, K.; Koyama, M.; and Tsukamoto, K. An improved colorimetric assay for interleukin 2. *J. Immunol. Methods* 1986; 93:157-165

38. Willey, R.L.; Smith, D.H.; Lasky, L.A.; Theodore, T.S.; Earl, P.L.; Moss, B.; Capon, D.J.; and Martin, M.A. *In vitro* mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. *J. Virol.* 1988; 62:139-147

39. Chen, L.L.; Frankel, A.D.; Harder, J.L.; Fawell, S.; Barsoum, J.; and Pepinsky, B. Increased cellular uptake of the human immunodeficiency virus-1 Tat protein after modification with biotin. *Anal. Biochem* 1995; 227:168-175

40. O'Brien. W.A.; Sumner-Smith, M.; Mao, S-H; Sadeghi, S.; Zhao, J-G; and Chen, I.S. Anti-human immunodeficiency virus Type 1 activity of an oligocationic compound mediated via gp120 V3 interations. *J. Virol.* 1996; 70:2825-2831

41. Brother, M.B.; Chang, H-K; Lisziewicz, J.; Su, D.; Murty, L.C.; and Ensoli, B. Block of Tat-mediated transactivation of tumor necrosis factor ß gene expression by polymeric-TAR decoys. *Virology* 1996; 222:252-256

42. Kashanchi, F; Reza, M.; and Brady, J.N. Inhibition of HIV-1 transcription and virus replication using soluble Tat peptide analogs. *Virology* 1997; 227:431-438

WHAT IS CLAIMED IS:

1. A peptide of the formula R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-$NH_2$ (SEQ ID NO:1), wherein R is the residue of a carboxylic acid, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof.

2. The peptide of Claim 1 wherein R is an acetyl group.

3. The peptide of Claim 1 wherein X is a cysteine residue.

4. The peptide of Claim 1 wherein X is a lysine residue.

5. The peptide of Claim 1 wherein Gln is replaced by Pro.

6. The peptide of Claim 1 which is N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Cys-(biotin)-$NH_2$ (SEQ ID NO:2).

7. The peptide of Claim 1 which is N-acetyl-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Lys-(biotin)-$NH_2$ (SEQ ID NO:3).

8. The peptide of Claim 1 which is N-acetyl-Arg-Lys-Lys-Arg-Arg-Pro-Arg-Arg-Arg-Cys(biotin)-$NH_2$ (SEQ ID NO:7).

9. A pharmaceutical composition comprising a peptide of the formula R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-$NH_2$ (SEQ ID NO:1) wherein R is the residue of a carboxylic acid, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, and a pharmaceutically acceptable carrier therefor.

10. The composition of Claim 9 wherein R is an acetyl group.

11. The composition of Claim 9 wherein X is a cysteine residue.

12. The composition of Claim 9 wherein X is a lysine residue.

13. The composition of Claim 9 wherein Gln is replaced by Pro.

14. The composition of Claim 9 adapted for parenteral administration.

15. A method of inhibiting HIV-1 replication which comprises administration of a peptide of the formula
R-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-X-(biotin)-$NH_2$ (SEQ ID NO:1)
wherein R is the residue of a carboxylic acid, and X is a Cys or Lys residue, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof.

16. The method according to Claim 15 wherein the peptide has the formula wherein R is an acetyl group.

17. The method according to Claim 15 wherein the peptide has the formula wherein X is a cysteine residue.

18. The method according to Claim 15 wherein the peptide has the formula wherein X is a lysine residue.

19. The method of Claim 15 wherein the peptide has the formula wherein Gln is replaced by Pro.

20. A peptide of the formula
R-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$.

wherein R is the residue of a carboxylic acid, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof.

21. The peptide of Claim 20, wherein R is an acetyl group.

22. A pharmaceutical composition comprising a peptide of the formula R-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$, wherein R is the residue of a carboxylic acid, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof, and a pharmaceutically acceptable carrier therefor.

23. The composition of Claim 22, wherein R is an acetyl group.

24. A method of inhibiting HIV-1 replication which comprises administration a pharamaceutical composition comprising a peptide of the formula R-DLys(ϵ-biotin)-DArg-DArg-DArg-DGln-DArg-DArg-DLys-DLys-DArg-$NH_2$, wherein R is the residue of a carboxylic acid, and analogs thereof, and the biologically and pharmaceutically acceptable salts thereof.

25. The method according to Claim 24, wherein the peptide has the formula wherein R is an acetyl group.

26. The method of claim 24, wherein the pharmaceutical composition is administered parenterally, transmucosally, transdermally, intramuscularly, intravenously, intradermaly, subcutaneously, intraperitonealy, intraventricularly, intracranialy or orally.

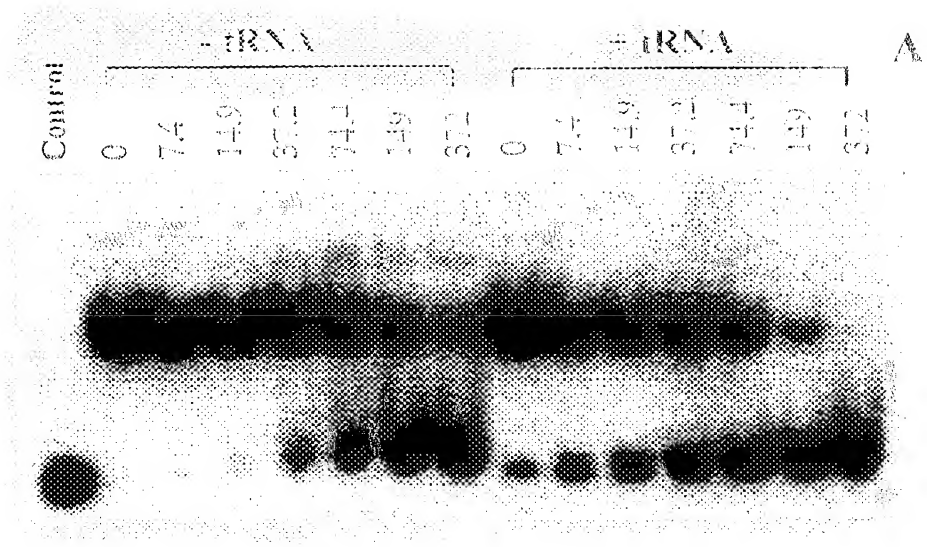


Figure 1A

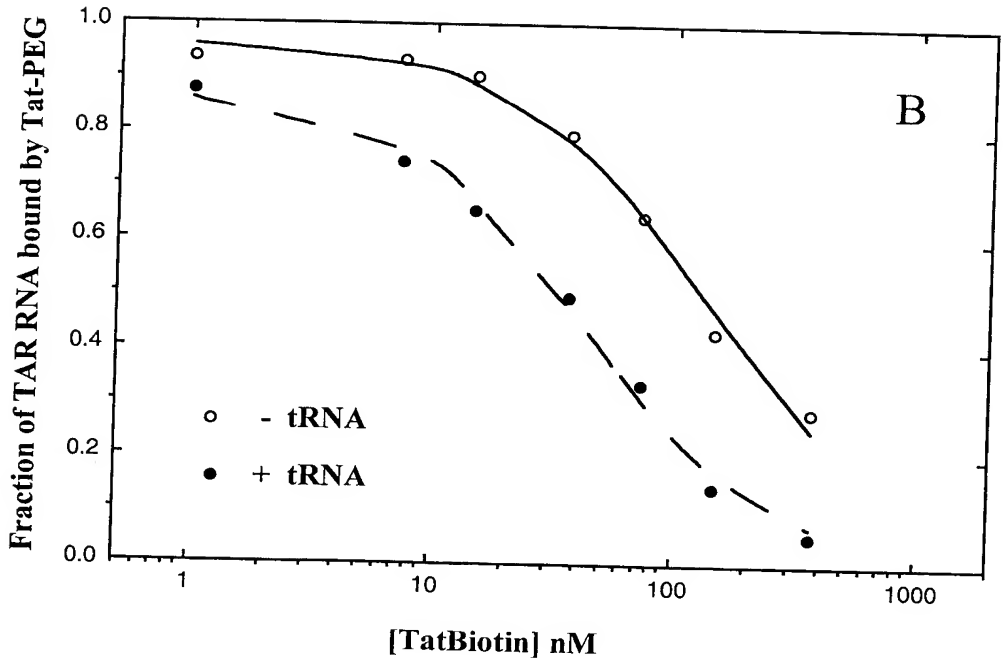
Fraction of TAR RNA bound by Tat-PEG
1.0
0.8
0.6
0.4
0.2
0.0
B
- tRNA
+ tRNA
1
10
100
1000
[TatBiotin] nM

Figure 1B

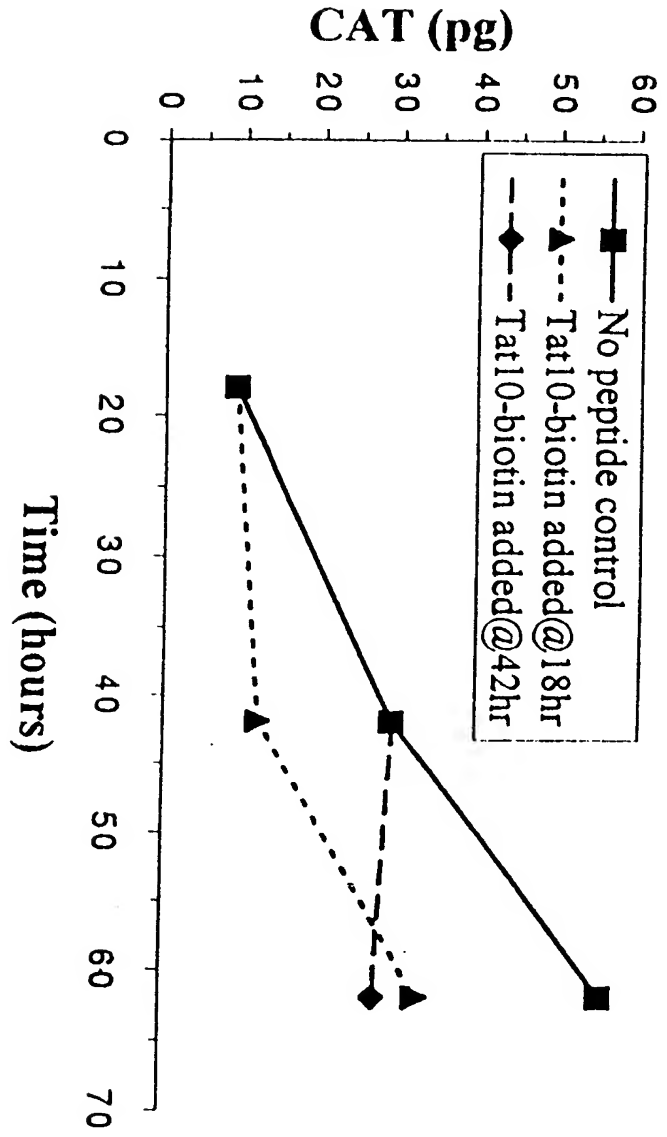
CAT (pg)
0
10
20
30
40
50
60
Time (hours)
0
10
20
30
40
50
60
70
No peptide control
Tat10-biotin added@18hr
Tat10-biotin added@42hr

Figure 2

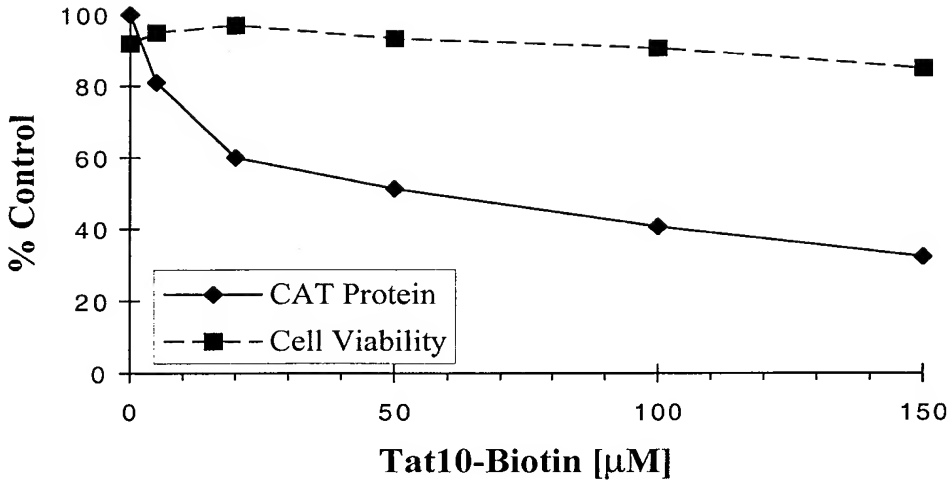
100
80
60
40
20
0
% Control
CAT Protein
Cell Viability
0
50
100
150
Tat10-Biotin [μM]

Figure 3

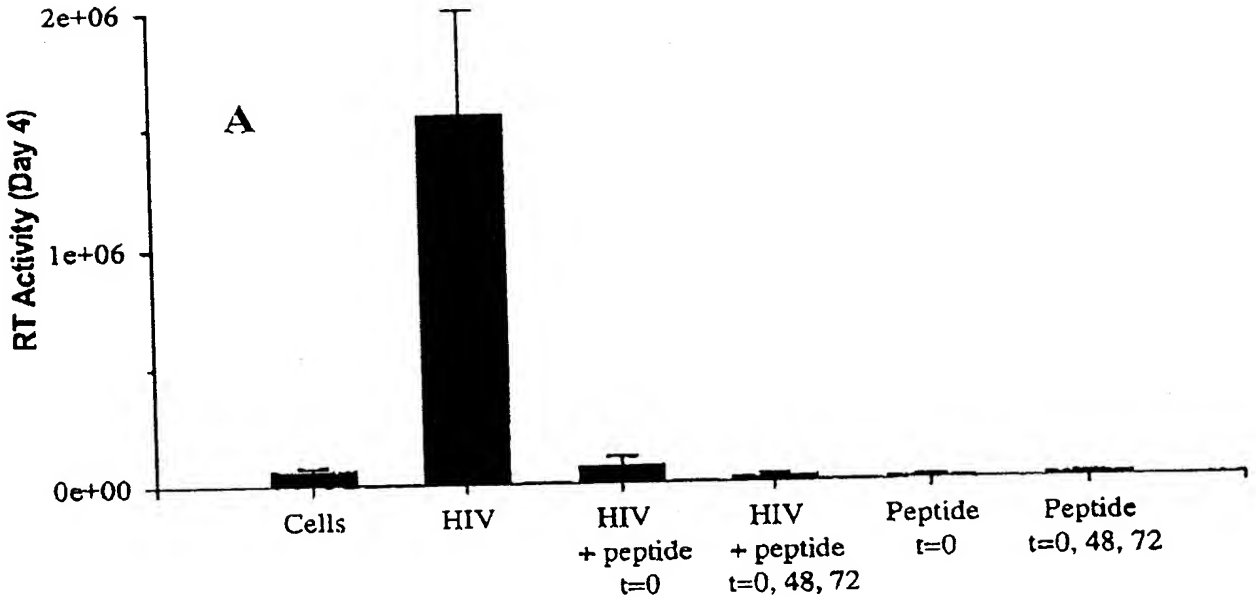


Figure 4A

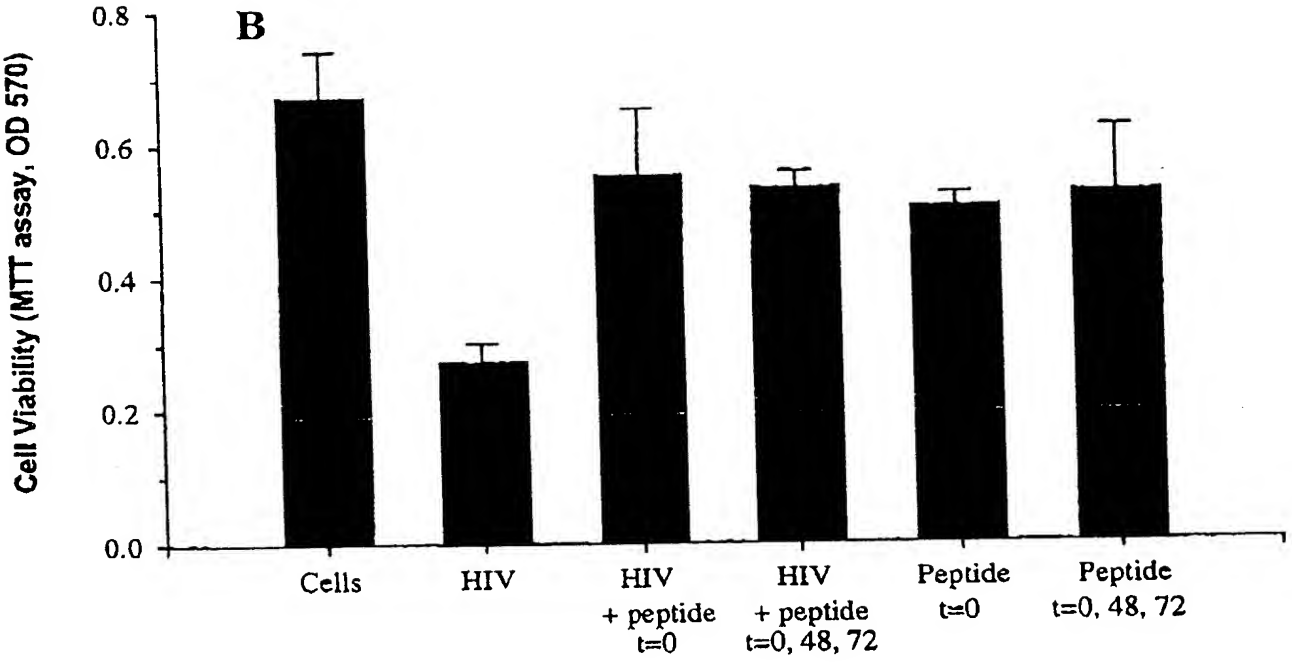


Figure 4B

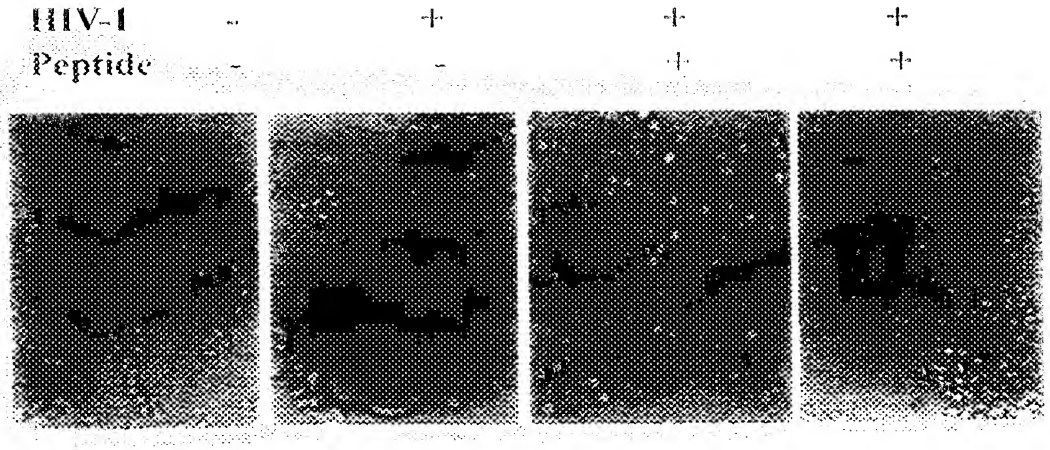


Figure 5

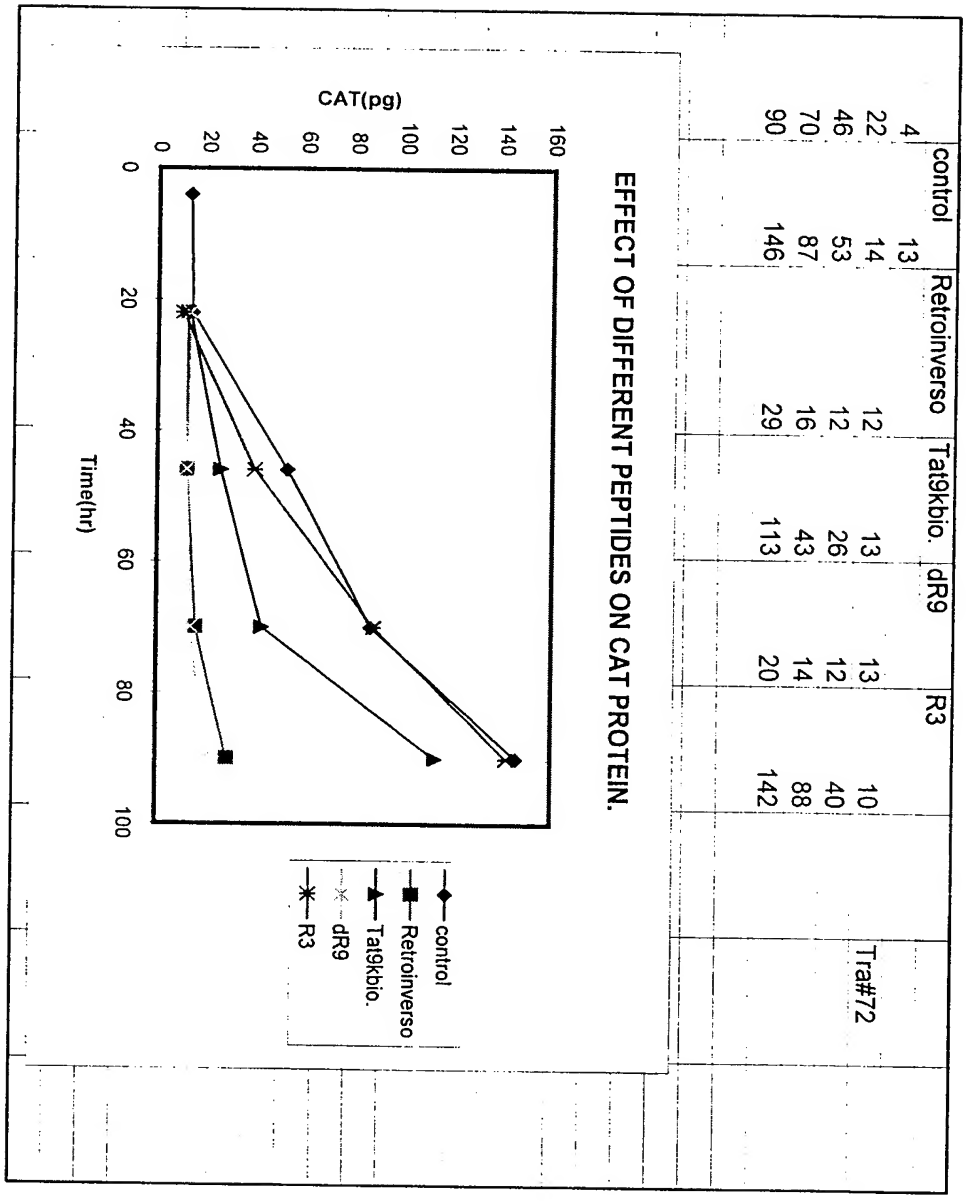
EFFECT OF DIFFERENT PEPTIDES ON CAT PROTEIN.
CAT(pg)
Time(hr)
0
20
40
60
80
100
120
140
160
control
Retroinverso
Tat9kbio.
dR9
R3
Tra#72
4
22
46
70
90
13
14
53
87
146
12
12
16
29
13
26
43
113
13
12
14
20
10
40
88
142

Figure 6

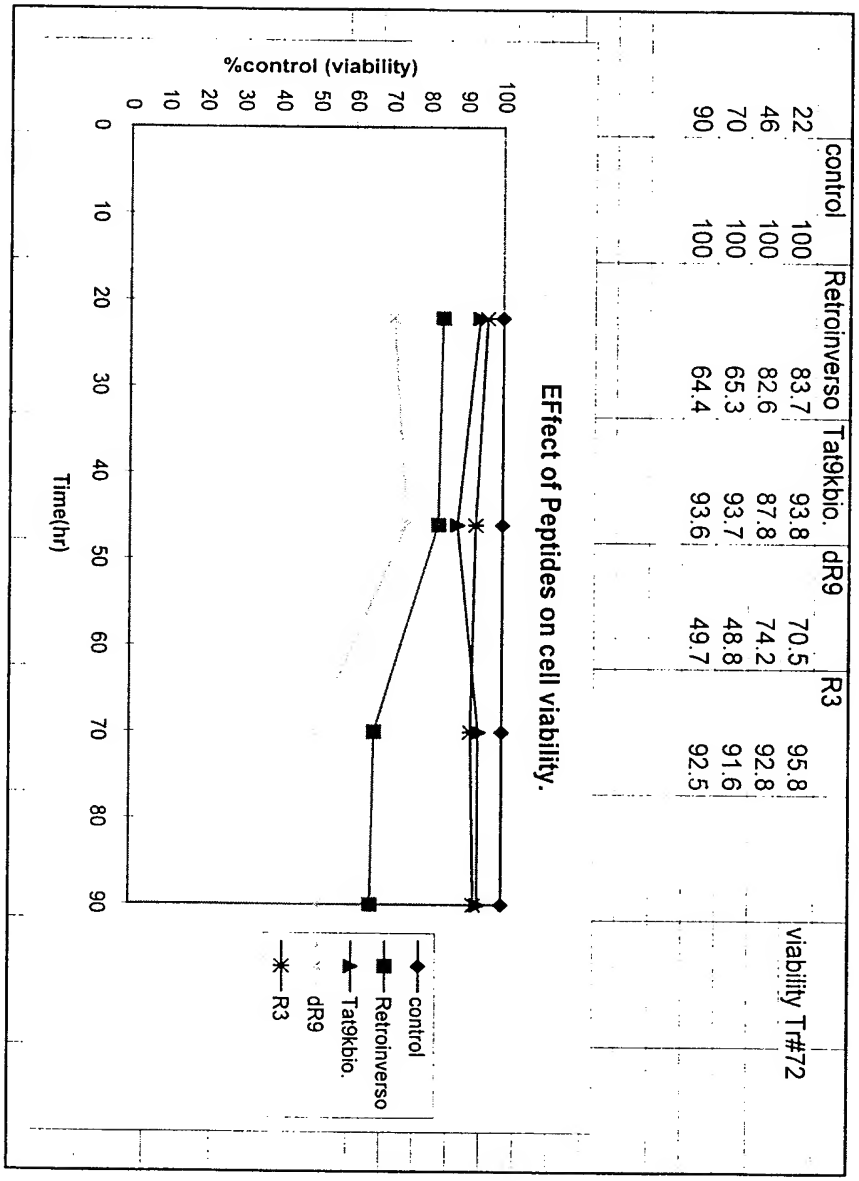
EFfect of Peptides on cell viability.
%control (viability)
Time(hr)
control
Retroinverso
Tat9kbio.
dR9
R3
viability Tr#72
control 100 100 100 100
Retroinverso 83.7 82.6 65.3 64.4
Tat9kbio. 93.8 87.8 93.7 93.6
dR9 70.5 74.2 48.8 49.7
R3 95.8 92.8 91.6 92.5
22
46
70
90

Figure 7